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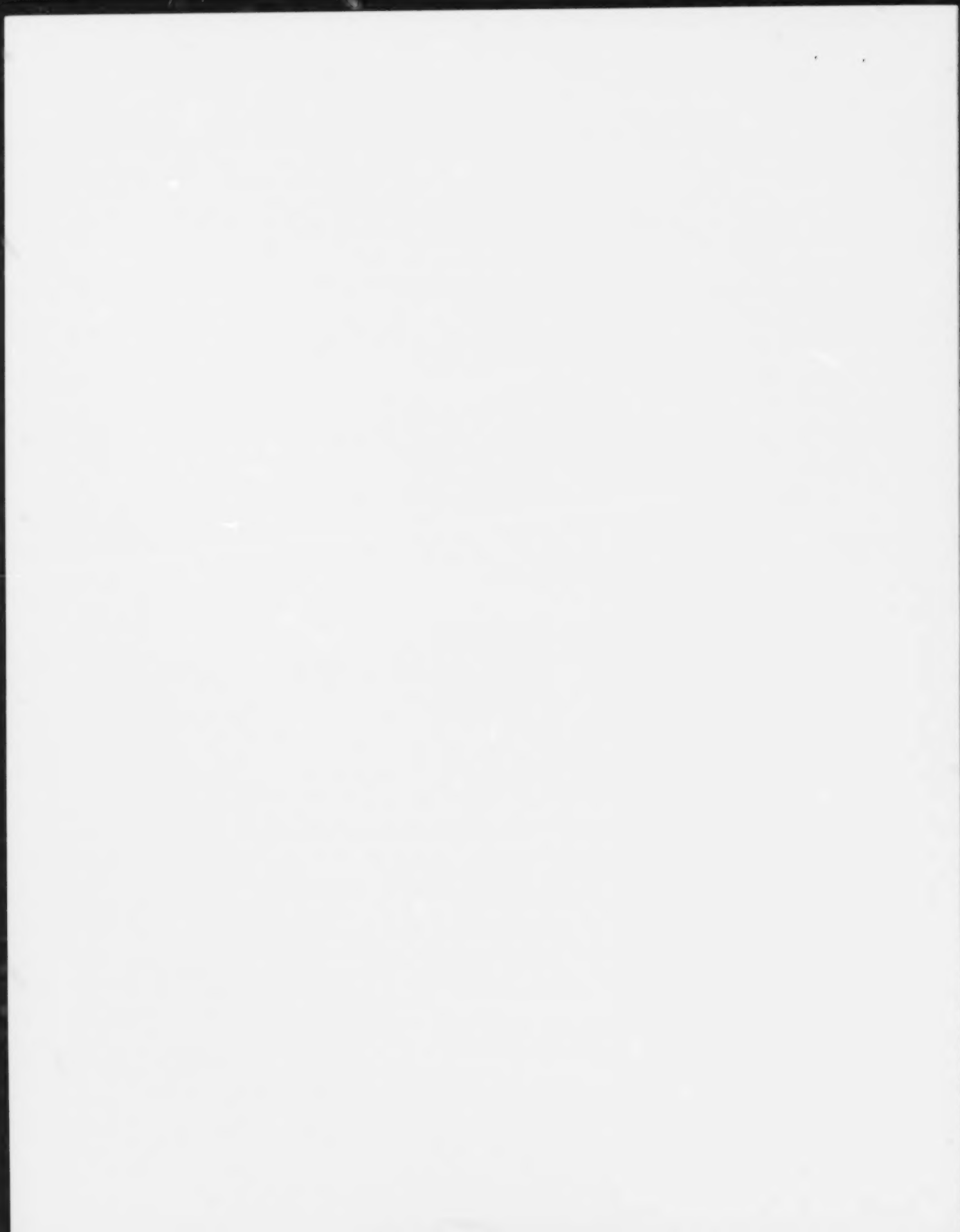
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**STUDY TO DEVELOP MEANS FOR MORE EFFECTIVE
CONTROL OF THE GOPHER POPULATION IN
SASKATCHEWAN**

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ADF Project # 200220076

Study to Develop Means for More Effective Control of the Gopher Population in Saskatchewan

Executive Summary

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1. Preamble

Richardson Ground Squirrels (*Spermophilus richardsonii*), more commonly known as gophers or prairie dogs, are considered pests and cause considerable agricultural and ecological damage. Gopher control in the province of Saskatchewan has been based largely on field application of rodenticides such as strychnine, zinc phosphide, and anticoagulants. For some time, there has been a general consensus among farmers that gopher control methods used in the province appear to be ineffective in reducing gopher densities (Saskatchewan Gopher Survey: Final Report, 2001). Thus, novel, more effective concepts of toxicological control of gophers need to be developed in order to address the problem of gophers in Saskatchewan.

The present project examined the enzymatic pathways responsible for systemic detoxification of xenobiotics in gophers. The primary goal was to identify metabolic features that could be exploited for the purpose of developing new, more effective species-specific, and ethically acceptable, technologies to control gopher populations.

1.1. Project Objectives

- To evaluate the main components of hepatic detoxification system of gophers using liver microsome fractions from previously exposed and naïve gophers.
- To characterize hepatic microsomal Cytochrome P450 system activity representing several key generic groups of biotransformation reactions with emphasis on aromatic hydroxylations, O-dealkylation, and N-dealkylation using selected compounds as probes.
- To evaluate the effects of inhibitor on the selected metabolic activities of Cytochrome 450 enzymes and determine IC₅₀ (concentration causing 50% inhibition).
- To evaluate the cytotoxicity of selected substrates using freshly isolated hepatocytes from gophers in the presence and absence of the inhibitor.

2. Experimental

2.1 Project Part I.

The first part of this project was focused on characterization of basic components involved in toxin metabolism in gophers. The results from this research were presented and discussed extensively in the document *Interim Report 2* submitted on February 25, 2005.

This data clearly demonstrated that all important components of the hepatic microsomal detoxification system were considerably higher in gophers that were previously exposed to toxins used for their chemical control. This indicates that gophers are capable of responding to toxicological challenge by increasing their detoxification potential.

The detoxification system in gophers has not been studied before. Hence, the present findings contributed a large body of new knowledge on the metabolism of toxins in gophers. The value of this contribution is important scientifically, and also is of significance from a practical point view. For instance, considering that the efficiency and detoxifying potential of hepatic mixed function oxidase system plays a crucial role in determining the toxicity of the ingested compounds, our data provided tangible evidence permitting a science based explanation for the remarkable ability of gophers to thrive despite constant attempts to control this pest using toxicological means over several decades.

2.2 Project Part II.

This part of the project was focused on 3 strategic areas: 1) study of potential metabolic weaknesses of the enzymatic pathways responsible for systemic detoxification of xenobiotics in gophers, 2) inhibition of enzymes mediating detoxification, and 3) toxicity tests using freshly isolated primary gopher hepatocytes.

2.2.1 An *in vitro* study of cytochrome P450 mediated detoxification activities:

Using validated HPLC methods, we quantified the *in vitro* cytochrome P450 enzyme-mediated activities for five substrates [coumarin and aniline aromatic hydroxylation, 7-methoxycoumarin O-demethylation, and N-methylaniline, N,N-dimethylaniline N-demethylation] in hepatic microsomes prepared from female and male naïve and toxin exposed gophers.

Male gophers (both naïve and exposed) were found to have considerably higher specific cytochrome P450 enzyme activities than both female groups; whereas, naïve gophers, regardless of gender, had higher activities than exposed gophers for all selected reactions. The lowest K_m values were for O-demethylation reactions, followed by aromatic hydroxylation reactions, whereas the highest K_m values were found for the N-demethylation reactions. The biotransformation of 7-methoxycoumarin demethylation showed the highest V_{max} value when normalized to cytochrome P450 content; whereas, biotransformation of coumarin aromatic hydroxylation had the lowest V_{max} value.

It is noteworthy that the metabolic capacity of the cytochrome P450 enzyme system was very variable within the studied gopher population. Following the evaluation of individual data points, two distinctive sub-groups were identified: 1) poor metabolizers, representing those having very low V_{max} values; and 2) stronger metabolizers, the remainder of the studied gopher population. In essence, the present study provided evidence that there is a relatively large number of individuals that show distinctive metabolic weaknesses in the metabolism of toxins.

2.2.2 Study of Cytochrome P450 inhibition:

The studied biotransformation reactions included coumarin and aniline aromatic hydroxylation, N-methylaniline and N, N-dimethylaniline N-demethylation, and 7-methoxycoumarin O-demethylation. In all cases, clotrimazole was found to be a potent inhibitor of biotransformation reactions mediated by the cytochrome P450 enzyme system in gophers.

2.2.3 Toxicity tests using freshly isolated gopher hepatocytes:

The cytotoxicity of several compounds (coumarin, aniline, N-methylaniline, N, N-dimethylaniline, and 7-methoxycoumarin) was tested using freshly isolated primary gopher hepatocytes. The results showed that each compound caused considerable cytotoxicity to gopher hepatocytes. However, the overall toxic effects were clearly different for different compounds. Coumarin and methoxycoumarin produced chronic toxicity characterized mainly by morphological changes in the hepatocytes. On the other hand, aniline, N-methylaniline, N, N-dimethylaniline caused acute cell death without overt morphological changes. It is noteworthy that the addition of inhibitor clotrimazole to primary hepatocyte cultures increased the cytotoxicity of all given compounds.

3. Summary of Pertinent Facts

- A very plausible explanation of why the current methods of gopher control are ineffective is the increased capacity of detoxification acquired upon exposure to toxin.
- The observations that there are gender differences in the metabolism of toxins, as well as the identification of a sub-population of poor metabolizers, are of practical significance in designing a strategy for gopher management.
- The findings that there are major differences in terms of detoxification kinetics between different compounds are of major significance, and can be exploited for the purpose designing better means of chemical control.
- The observations from the isolated enzyme system, as well as using intact hepatocyte culture, provided evidence that the concept of using inhibitors to increase the toxic effect of compounds used for gopher control is valid from a practical application stand point.

4. Concluding Remarks

The findings from the present project provided solid base for the second phase of research with emphasis on the following key practical aspects of gopher control method development: 1) work on a generic structure of putative toxins and detoxification inhibitors, 2) preparation of the prototype, and 3) basic toxicological study using cultured hepatocytes followed by field application testing.



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ADF Project # 200220076
Study to Develop Means for More Effective Control of the Gopher Population in Saskatchewan

Final Report, December 27, 2005

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Preamble: The underlying idea behind the inception of this project was to develop science based knowledge that may help in the development of better means of toxicological control of gophers. The present project had two closely related goals: 1) educational, and 2) scientific. I am very pleased to report that both goals were accomplished to the fullest extent. In the first instance, this work developed a highly qualified professional, and in the second instance this project generated high quality scientific material that will be published in peer reviewed journals.

1. EDUCATION

The graduate student working on this project, Ms. Binbing Ling, completed all educational requirements set out for this project in a timely manner and with high marks. The Advisory Committee noted that the student demonstrated a high degree of academic performance. An important educational outcome of this project was a very high quality thesis.

The student presented a very interesting seminar on her work. Defense of her thesis took place on November 30, 2005. Dr. David M. Janz, Department of Biomedical Sciences and Toxicology Centre, was an external examiner. The external examiner and the Advisory Committee deliberated the scientific merit of the thesis submitted by the candidate and noted that it is of very high quality. Following a discussion *in camera*, the external examiner and the Committee members unanimously recommended acceptance of the thesis as partial fulfillment of the requirements for the degree of Master of Science in the Department of Animal and Poultry Science, University of Saskatchewan. The thesis were submitted to the college of Graduate Studies and Research on December 15, 2005.

2. EXPERIMENTAL

2.1 Project Part I

Background: The first part of this project was focused on the characterization of basic components involved in toxin metabolism in gophers. This part of the project generated an abundance of novel and important scientific data. It is of interest to mention that prior to our study, there was no scientific information on the subject of toxin metabolism in gophers. The results from this research were presented and discussed extensively in the document *Interim Report 2* submitted on February 25, 2005. The key findings from this study are summarized in Figure 1.

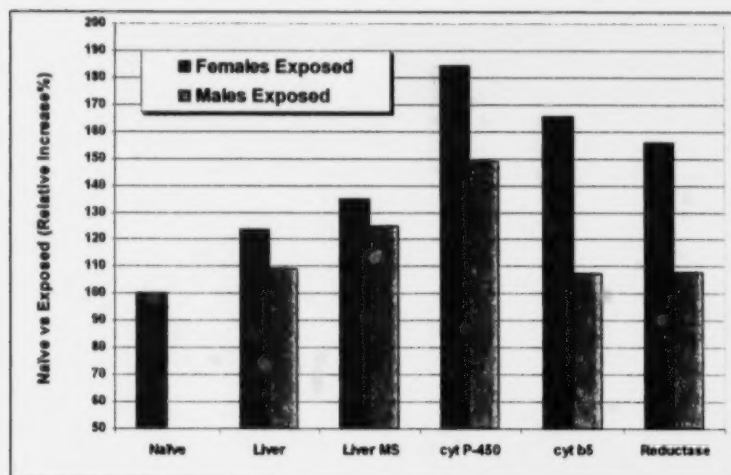


Figure 1. Relative differences in various components of the hepatic cytochrome P-450 system between naïve and exposed gophers. Red bar on the graph indicates basic activity of naïve gophers (100%), and this is compared to relative increases for exposed males (green bars) and females (blue bars).

From the comparative data presented in Figure 1, it is apparent that all important components of the hepatic microsomal detoxification system were considerably higher in gophers that were previously exposed to toxins used for their chemical control.

The efficiency and detoxifying potential of cytochrome P450 hepatic mixed function oxidase system plays a crucial role in determining the magnitude and duration of toxic effects. In this context, from the data presented above it is evident that gophers have tremendous capacity to increase their detoxifying power when exposed to toxins. This may explain the remarkable ability of gophers to thrive, despite constant attempts to control this pest using toxicological means over several decades.

Part II of the project was focused on 3 strategic areas: 1) toxin metabolism with emphasis on potential metabolic weaknesses of the enzymatic pathways responsible for systemic detoxification of xenobiotics in gophers, 2) concept of enzyme inhibition as a potential means to decrease the detoxification potential, and thus enhance the efficacy of poisons used for gopher control, and 3) toxicity tests at cellular level using freshly isolated primary gopher hepatocytes.

2.2 Project Part II

2.2.1. *In Vitro* Metabolism of Selected Substrates by the Hepatic Microsomal Cytochrome P450 System in Gophers

Background: For chemical control of pest animals, the most practical delivery method for pesticides is through ingestion. In this context, while considering the effectiveness of toxins, it is important to consider two critical facts: 1) liver is the primary organ involved in the detoxification of orally administered toxins, and 2) the magnitude and duration of toxic effects greatly depends on the catalytic activities of hepatic biotransformation enzymes. For these reasons, a better understanding of the hepatic detoxification mechanisms in gophers may provide valuable information that may help in the design of more effective chemical means of gopher population management. Accordingly, our primary objectives were to evaluate the activity of major detoxification pathways of the hepatic cytochrome P450 system.

Selected compounds were used as generic probes to evaluate the kinetics of major biotransformation reactions *in vitro*. Coumarin and aniline were selected as the probes to examine aromatic hydroxylation. The activity of enzymes involved in N-dealkylation was evaluated using N,N-dimethylaniline and N-methylaniline, and O-demethylation pathways were examined using 7-methoxycoumarin as a probe.

Experimental Strategy and Rationale: The experimental strategy employed in the present study was based on the evaluation of the key kinetic parameters (K_m and V_{max}) of detoxification reactions. V_{max} is a value of maximum velocity of the enzymatic reaction, whereas K_m represents concentration of the substrate required to allow enzymatic reaction to proceed at 50% of maximum velocity. In other words, low V_{max} values indicate relatively low rates of detoxification. Such toxins that are metabolized at low rate will accumulate in the body and cause adverse effects, and eventually death. High K_m values indicate a poor affinity of the toxin for that particular cytochrome P450 pathway and high toxin concentrations are needed to reach detoxification rates at 50% of maximal velocity. This allows the existing toxin to distribute at high levels throughout the body causing adverse effects and possibly death.

The logistic rationale for this approach can be illustrated in the following table.

Kinetic Parameters	Toxin with high inherent toxicity		Toxin with low inherent toxicity	
	Metabolites: Low or (High) Toxicity		Metabolites: High toxicity	
	Level	Expected Toxic Effect	Level	Expected Toxic Effect
V_{max}	High	Low (High)	High	High
	Low	High	Low	Low
K_m	High	High	High	Low
	Low	Low	Low	High

Knowledge of the basic parameters of detoxification kinetics is of practical importance. For instance, if a compound has a high inherent toxicity, and metabolites have low toxicity, poor detoxification efficiency of this compound may indicate a higher potential for toxicity. Hence, if the detoxification pathways *in vitro* demonstrate low V_{max} and high K_m values, there is a strong potential for significant

in vivo toxicity. On the other hand, compounds with low inherent toxicity, but with highly toxic metabolites, kinetics *in vitro* showing high V_{max} values and low K_m values indicate high potential for *in vivo* toxicity. High V_{max} indicate a rapid turnover of toxin to its toxic metabolites, whereas low K_m indicate that even though a small amount of toxin is introduced into the body, it can be rapidly transformed into toxic metabolites. Depending upon the rate of metabolite elimination, toxic metabolites may accumulate in the body and contribute to high toxicity.

Materials and Methods (for details see appendix A)

Results

Aromatic Hydroxylation: There were no significant differences for hydroxylation of coumarin or aniline with respect to either exposure status or gender. However, naïve gophers tended to have higher K_m values than exposed gophers for both females and males. Naïve gophers had higher V_{max} values than exposed ones when normalized to cytochrome P450 content for both females and males, whereas exposed gophers tended to have higher activity per unit of body weight than naïve gophers for both genders.

Dealkylation Reactions

N-methylaniline metabolism: The K_m values of naïve gophers are higher than exposed gophers for both sexes, but the differences were not statistically significant. There was a significant difference when comparing K_m value within genders regardless of toxin exposure status ($P<0.03$). There was a statistical difference between naïve and exposed males for the V_{max} values based on the cytochrome P450 content. The effects of exposure status in males tended to be higher than that in females for both specific activity ($P=0.07$) and whole body activity ($P=0.06$).

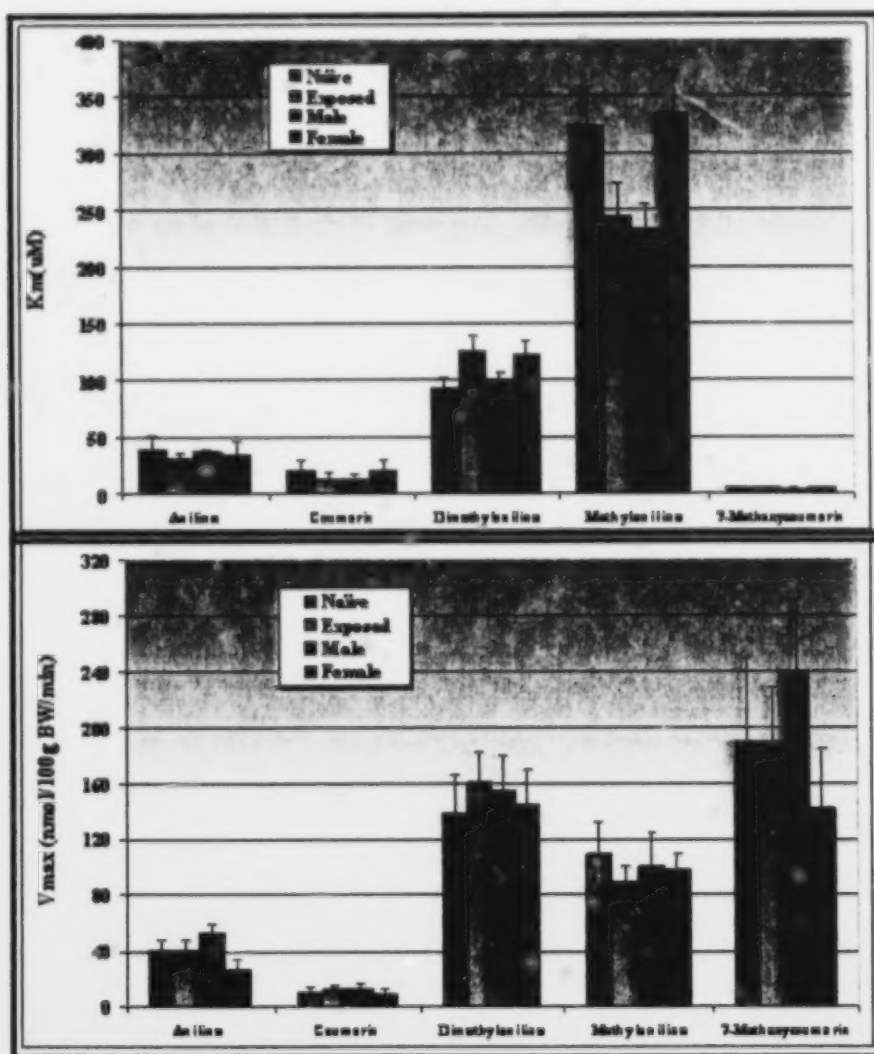
N,N-dimethylaniline metabolism: The K_m values for naïve gophers were lower than the exposed gophers with respect to both sexes and the difference between naïve and exposed females was statistically significant ($P<0.05$). When only considering exposure status, statistically significant differences in the K_m values existed between naïve and exposed gophers ($P<0.04$). There were no statistically significant differences for V_{max} values based on cytochrome P450 content between exposed and naïve female gophers. However, the difference between naïve and exposed male gophers was statistically significant. The effects of exposure status in females tended to be higher than that of males for K_m values ($P=0.07$). The effect of exposure status on males was significantly higher than females for specific activity ($P=0.03$). However, no statistically significant difference existed between the effect of status on genders for the whole body activity ($P=0.05$).

7-methoxycoumarin metabolism: There were no statistically significant differences for K_m values for all four groups. The V_{max} values based on cytochrome P450 content for naïve gophers were higher than exposed gophers with respect to both sexes, although the difference was not statistically significant. Nevertheless, when calculated on the basis of whole body activity, exposed females had higher V_{max} values than naïve females, and, exposed males had slightly lower V_{max} values than naïve males. When considering the population of males and females together, there was no difference between naïve gophers and exposed ones ($P=1.00$). No significant effects of status on gender were observed for both K_m and V_{max} .

Comparison of Differences in Metabolism of Various Substrates

Biotransformation Enzymes Kinetics: The comparative data for the studied substrates are summarized in Figure 2 and Table 1.

Figure 2. Comparison of cytochrome P450 K_m and V_{max} values in naïve vs exposed, and males vs females gophers.



The K_m values were highest for N-methylaniline and lowest for 7-methoxycoumarin kinetics regardless of status and gender differences. Overall, the dealkylation reactions showed considerably higher V_{max} than aromatic hydroxylation reactions. The trend for the specific activity was similar as for the whole body activity.

Table 1. Comparison of cytochrome P450 kinetics for different substrates: affinity, specific activity, and whole body activity in exposed and naïve gophers (Mean $\pm$ SE).

Substrate	K_m (μM)		V_{max} (nmol/nmol/CYP450/min)		V_{max} (nmol/100g BW/min)	
	Exposed	Naïve	Exposed	Naïve	Exposed	Naïve
Coumarin	13.00 $\pm$ 5.05 ^a	20.65 $\pm$ 8.74 ^a	0.23 $\pm$ 0.05 ^a	0.33 $\pm$ 0.11 ^a	12.70 $\pm$ 2.85 ^a	10.48 $\pm$ 3.57 ^a
Aniline	31.60 $\pm$ 4.61 ^a	39.19 $\pm$ 12.24 ^a	0.75 $\pm$ 0.13 ^a	1.23 $\pm$ 0.17 ^a	40.83 $\pm$ 7.96 ^{ab}	40.21 $\pm$ 7.30 ^{ab}
NMA	243.58 $\pm$ 29.98 ^b	324.15 $\pm$ 34.30 ^b	1.78 $\pm$ 0.28 ^c	3.29 $\pm$ 0.48 ^b	89.51 $\pm$ 12.16 ^b	109.75 $\pm$ 22.91 ^{bc}
DMA	126.35 $\pm$ 12.97 ^c	93.98 $\pm$ 8.08 ^c	3.07 $\pm$ 0.28 ^b	4.14 $\pm$ 0.55 ^{bc}	160.53 $\pm$ 21.72 ^c	138.02 $\pm$ 28.95 ^c
7-MC	4.01 $\pm$ 1.01 ^a	3.85 $\pm$ 0.37 ^a	3.46 $\pm$ 0.62 ^b	5.24 $\pm$ 1.30 ^c	190.26 $\pm$ 38.17 ^c	190.22 $\pm$ 60.15 ^c

NMA= N-methylaniline, DMA= N,N-dimethylaniline, 7-MC=7-methoxycoumarin. The values are means of 12 replications (SE=standard error). Means were compared using Fisher's LSD, α = 0.05. Means with the same letters are not significantly different.

Several points of interest in differences in enzyme kinetics parameters are noteworthy. For instance, the K_m values of aniline were 2.1-fold higher than the K_m values of coumarin. Furthermore, specific activity was 3.5-fold higher, even though variations existed between different groups for both substrates. The K_m value of N-methylaniline was 2.6 times higher than N,N-dimethylaniline, whereas the V_{max} values for N,N-dimethylaniline were 1.4-fold higher than for N-methylaniline.

N-methylaniline metabolism showed the highest K_m value in both exposed and naïve gophers, which significantly differed from the other substrates studied. There were no significant differences in K_m values among coumarin, aniline, and 7-methoxycoumarin, but the K_m values for N-demethylation were significantly higher than for either aromatic hydroxylation or O-demethylation. Microsomal Cytochrome P450 enzymes tended to show the highest specific activity and whole body activity for O-demethylation in both exposed and naïve gophers, whereas enzymes responsible for coumarin hydroxylation tended to have the lowest specific activity and whole body activity in both exposed and naïve gophers.

N-demethylation reactions showed significantly higher specific activity in both exposed and naïve gophers than aromatic hydroxylation, and O-demethylation had significantly higher specific activity and whole body activity than aromatic hydroxylation in all gophers.

Intrinsic Clearance: For all substrates examined in the present study, naïve gophers tended to have higher intrinsic clearance than the gophers previously exposed to toxins (Table 2). However, 7-methoxycoumarin had the highest intrinsic clearance value when considering all the experimental gophers as one group as compared with the other compounds. N-methylaniline had the lowest intrinsic clearance value.

Table 2. Comparison of microsomal intrinsic clearance values analyzed by enzyme kinetic method: $Cl_{int} = V_{max} / K_m$ for selected compound between naïve gophers and gophers exposed previously to toxins.

Substrate	Intrinsic clearance ($\mu\text{M}/\text{min}/\text{g}$ protein)		
	Exposed n = 12	Naïve n = 12	Whole Population n = 24
Coumarin	31.40 $\pm$ 7.86 ^a	37.24 $\pm$ 16.99 ^a	34.32 $\pm$ 9.18 ^a
Aniline	22.72 $\pm$ 4.54 ^a	29.42 $\pm$ 4.38 ^a	26.07 $\pm$ 3.17 ^a
NMA	6.34 $\pm$ 0.56 ^a	8.10 $\pm$ 1.48 ^a	7.22 $\pm$ 0.80 ^a
DMA	22.42 $\pm$ 1.81 ^a	35.05 $\pm$ 6.22 ^a	28.73 $\pm$ 3.43 ^a
7-MC	977.35 $\pm$ 172.18 ^a	978.83 $\pm$ 207.47 ^a	978.09 $\pm$ 131.84 ^a

NMA= N-methylaniline, DMA= N,N-dimethylaniline, 7-MC=7-methoxycoumarin. Values are means $\pm$ standard error. Means were compared using Fisher's LSD, $\alpha = 0.05$. Means with the same letters are not significantly different.

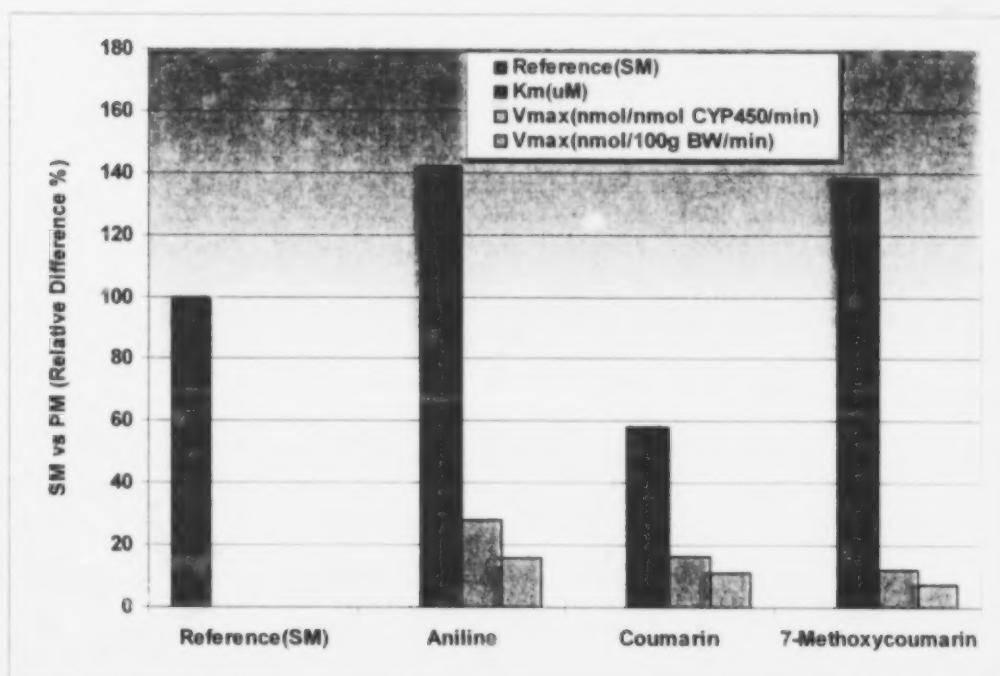
It is noteworthy that there were considerable differences among substrates in terms of intrinsic clearance. This may provide valuable information for screening the candidates of toxins that can be potentially used for gopher control.

Analysis of the Population

There was a substantial variability in the kinetic parameters of cytochrome P450 enzymes involved in coumarin, aniline and 7-methoxycoumarin biotransformation. However, closer examination of the individual data points revealed several interesting trends. The analysis of the population data showed clearly identifiable subgroups with distinctly different metabolic characteristics. The sub-group of individuals showing less than 20% of the highest value of V_{max} based on 100g BW was characterized as poor metabolizers (PM), whereas the remainder of the population was defined as strong metabolizers (SM).

Notably, 42% of the sample population could be described as PM group for coumarin, 25% for aniline and 29% for 7-methoxycoumarin. Enzymes responsible for aniline and 7-methoxycoumarin metabolism in PM had slightly lower affinity (higher K_m) in comparison to SM. However, the enzymes responsible for coumarin metabolism differed in that PM had slightly lower affinity than the enzymes in SM. From the comparative data of V_{max} , it was apparent that both specific activities and whole body activities of coumarin, aniline, and 7-methoxycoumarin metabolism in PM were considerably lower than those in SM. Furthermore, the extent of differences between whole body activities in PM and SM were much higher than for the specific activity (Figure 3).

Figure 3. Relative differences in K_m and V_{max} values between poor metabolizers (PM) and strong metabolizers (SM). Red bar on the graph indicates K_m and V_{max} for the SM (100%), which is then compared to relative values for K_m and V_{max} for specific activity and whole body activity respectively.



Comments: In general, the present study demonstrated that naïve male gophers have higher enzyme activities than naïve females for a given biotransformation pathway, although these differences were not statistically significant. The data suggest that naïve male gophers likely have higher rates of xenobiotic metabolism *in vivo* than naïve females. Interestingly, naïve female gophers had 16.8 % less total cytochrome P450 content than naïve male gophers (for details see Interim Report #2). This difference in total cytochrome P450 content may help to explain why naïve female gophers metabolize selected compounds at a different rate than naïve male gophers.

The observed differences associated with gender in the present study are consistent with the findings in other species. Sex dependent differences in biotransformation of chemicals have been found in other rodent species. For instance, male rats have been found to have higher rates of xenobiotic metabolism than female rats (Kedderis and Mugford, 1998).

There is little doubt that previous exposure to toxins influenced the content and activity of cytochrome P450 enzymes in gophers. Exposed gophers demonstrated higher total cytochrome P450 enzyme content, but in some instances, showed lower specific activities of bio-transformation reactions for some of the studied substrates than naïve gophers. These findings do not come as a surprise. The exposed gopher population in the present study was treated with strychnine, and therefore the cytochrome P450 enzymes in these gophers were induced in response to strychnine. Hence, in exposed gophers, because the bulk of cytochrome P450 enzymes increased, the relative component of bio-transformation reactions activities of some specific enzymes may appear to be lower.

$V_{\max}$ and K_m are the two parameters that define the kinetic parameters of enzymatic reaction as a function of substrate concentration. K_m is an approximate measure of the affinity of the substrate for the enzyme and $V_{\max}$ is the maximum rate of an enzyme catalyzed reaction (i.e. when the enzyme is saturated by the substrate). K_m values for N-methylaniline were found to be the highest among the selected substrates for both naïve and exposed gophers, whereas, K_m values for 7-MC were the lowest. Also, N-demethylation enzymes had a higher K_m value than aromatic hydroxylation enzymes. High K_m value indicates that the substrate must reach a higher concentration before it is metabolized effectively. If the inherent toxicity of such a substrate is high, systemic toxicity can occur before the compound is metabolized at a toxicologically meaningful rate. Thus, in gophers, compounds that undergo detoxification via N-demethylation pathways would be expected to have higher potential to cause a toxic effect than compounds that are detoxified via aromatic hydroxylation pathways.

Of note, metabolism of coumarin, aniline, and 7-methoxycoumarin was highly variable. Variability of catalytic activity of cytochrome P450 enzymes is a common characteristic of this enzyme system, and this is mainly associated with a large range of variability in the expression of enzyme protein (Lin and Lu, 2001). This feature has also been identified in the present study in gophers. Interestingly, $V_{\max}$ values varied between individuals by as much as 180 fold for coumarin, and 50 fold for aniline and 7-methoxycoumarin. Yet, there was a weak correlation between the individual cytochrome P450 content and their catalytic activity in our study. This can be explained by the fact that the activities of various metabolic pathways examined in our study were comprised of the sum of activities of different cytochrome P450 isoforms.

As a result of variation in metabolism, the metabolic conversion and excretion rate of toxins would be expected to vary among individuals. This is normal for the xenobiotic metabolism catalyzed by cytochrome P450 system (McKinnon and Evans 2000). In the present study, it was apparent that both specific activities and whole body activities of coumarin, aniline, and 7-methoxycoumarin metabolism in poor metabolisers were considerably lower than in strong metabolisers. Furthermore, the extent of these differences between whole body activities in PM and SM were much higher than the specific activity. This observation is of practical significance, because the knowledge of specific metabolic characteristics in gophers will be advantageous for developing a potent gopher control method. This issue will be further addressed later.

Another important observation from this project that is of practical importance is that there are large differences in intrinsic clearance of different compounds. The parameter that determines the duration of toxicity of a given compound is systemic clearance (Cotreau et al., 2005). Hence, the concept of intrinsic clearance (CL_{int}) is very useful, because it allows us to extrapolate the *in vitro* kinetic data to metabolic activity *in vivo*, where liver microsomal intrinsic clearance values can be used to predict hepatic clearance (Lin, 1998). For compounds with low intrinsic clearance values, the elimination rate of the compounds depends on enzymatic activity. This means enhancement in hepatic enzyme activity, resulting from the addition of an inducer, will have a more profound effect on clearance of such compounds. Conversely, enzyme inhibitors will have the opposite effect.

2.2.2. Study on Inhibition of Selected Detoxification Pathways

Background: Numerous compounds may alter the activity of the cytochrome P450 system, with the net result being either enhancement, or inhibition, of the metabolism of other compounds. Imidazole derivatives are among the most potent of inhibitors cytochrome P450 (Turan et al., 2001). These inhibitors interact with microsomes binding tightly with the prosthetic heme iron of cytochrome P450

proteins. Because the heme prosthetic group is the oxidation center for cytochrome P450 catalyzed reactions, these compounds are competitive cytochrome P450 inhibitors. Clotrimazole (CTZ) is a potent specific inhibitor of cytochrome P450 enzymes (Draper et al., 1997).

In this study, the inhibitory potential of clotrimazole on selected cytochrome P450 catalyzed reactions was evaluated in gopher hepatic microsomes cytochrome P450 selected phase I biotransformation pathways. The studied reactions included aromatic hydroxylation using aniline, N-demethylation using N-methylaniline and N,N-dimethylaniline, and O-demethylation using 7-methoxycoumarin as probe substrates.

Materials and Methods (for details see appendix A)

Results

The results of inhibition reactions for all substrates tested in the present study are summarized in Table 3.

Table 3. Inhibition of cytochrome P450 enzyme reactions by clotrimazole in hepatic microsomal preparations from gophers. Data represent the mean $\pm$ SE from 6 gophers.

Subject	IC 50 (μ M)			
	NMA	DMA	7-MC	Aniline
Females Naïve	0.24 $\pm$ 0.07 ^a	0.34 $\pm$ 0.20 ^a	0.39 $\pm$ 0.13 ^{ab}	2.10 $\pm$ 2.06 ^{ab}
Females Exposed	0.21 $\pm$ 0.03 ^a	0.57 $\pm$ 0.28 ^a	0.48 $\pm$ 0.08 ^a	3.78 $\pm$ 2.21 ^a
Males Naïve	0.22 $\pm$ 0.13 ^a	0.67 $\pm$ 0.24 ^a	0.79 $\pm$ 0.09 ^{ab}	0.27 $\pm$ 0.14 ^b
Males Exposed	0.17 $\pm$ 0.06 ^a	0.71 $\pm$ 0.25 ^a	0.76 $\pm$ 0.18 ^b	0.45 $\pm$ 0.18 ^b
ANOVA (gender)	<i>P</i> =0.74	<i>P</i> =0.72	<i>P</i> <0.02	<i>P</i> <0.03
ANOVA (status)	<i>P</i> =0.63	<i>P</i> =0.33	<i>P</i> =0.83	<i>P</i> =0.49
ANOVA (gender*status)	<i>P</i> =0.91	<i>P</i> =0.40	<i>P</i> =0.64	<i>P</i> =0.39

NMA= N-methylaniline, DMA= N,N-dimethylaniline, 7-MC=7-methoxycoumarin. IC 50 = concentration of the inhibitor required to reduce the reaction to 50% of V_{max} . The values are means of six replications SE=standard error. Means were compared using Fisher's LSD, α = 0.05. Means with the same letters are not significantly different.

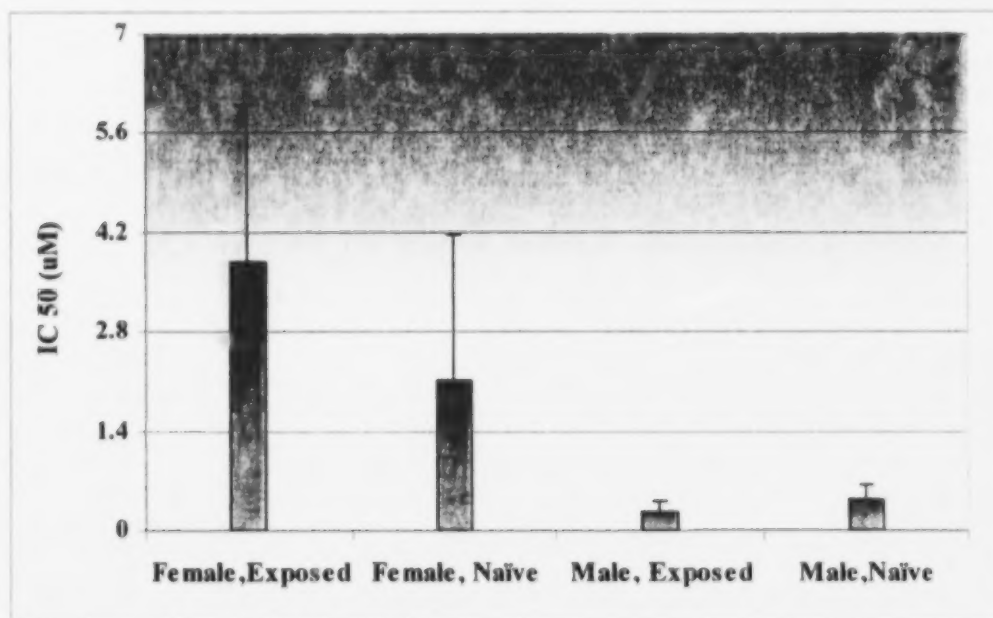
Notably, the characteristics of inhibition in terms of IC₅₀ varied between different substrates. There were no statistically significant differences in IC₅₀ values with respect to gender or status for N-methylaniline metabolism. However, IC₅₀ values of naïve gophers for both females and males were higher than exposed gophers. Female gophers tended to have higher IC₅₀ values than male gophers within naïve and exposed categories. Thus, for N-methylaniline metabolism, clotrimazole appeared to be a more potent inhibitor in exposed gophers than in naïve gophers.

There were no statistically significant differences in IC_{50} values with respect to both gender and status for N,N-dimethylaniline metabolism. However, there were still trends that were found for the inhibitory potency of clotrimazole. IC_{50} values of naïve gophers tended to be lower than exposed gophers for both females and males, which tended to be higher for males than for females for both naïve and exposed gopher groups.

There were statistically significant differences for IC_{50} values for 7-methoxycoumarin inhibition between females and males within the group of exposed gophers. Considering both naïve and exposed gophers as one population, there was also a statistically significant difference for IC_{50} values between genders where male gophers had higher IC_{50} values than female gophers ($P < 0.02$).

There were no statistically significant differences in IC_{50} values between naïve and exposed gophers of the same gender for aniline inhibition, but the IC_{50} values for female exposed gophers were higher than exposed and naïve males ($P < 0.05$). Furthermore considering both naïve and exposed gophers as one group, there were statistically significant differences for IC_{50} values between gender; where female gophers had higher IC_{50} values than male gophers ($P < 0.03$). Also, the IC_{50} values of exposed female gophers were found to be higher than naïve female gophers (Figure 4).

Figure 4. IC_{50} of CTZ for cytochrome P450 catalyzed aromatic hydroxylation of aniline in the four groups of gopher hepatic microsomal preparations (n=6).



Comments: The most potent inhibition occurred with N-demethylation and least potent inhibition with aromatic hydroxylation. Furthermore, status played an important role in the inhibitory potency of clotrimazole on the biotransformation of selected compounds. For N-methylaniline metabolism, clotrimazole is more potent in exposed gophers than naïve gophers for both females and males. However, for aniline, 7-methoxycoumarin and N,N-dimethylaniline, clotrimazole is more potent in naïve gophers than in exposed gophers, but it is slightly more potent in exposed males than in naïve males for 7-methoxycoumarin biotransformation. In addition to exposure status, gender was another

factor that influenced the potency of clotrimazole. Clotrimazole has higher potency in females for N-methylaniline and aniline metabolism. However, the potency in males was higher than in females for N,N-dimethylaniline metabolism as well as 7-methoxycoumarin.

Identification of compounds that inhibit the cytochrome P450 enzymes in gophers increases the number of options available for designing a more efficient species-specific method to control the gopher population. As discussed in the previous section, the inhibition of enzymes will result in a considerable enhancement in the toxicity of pesticide. The oral co-administration of pesticide with a potent inhibitor would result in a synergistic effect by increasing toxicity by the inhibition of first-pass metabolism and a reduction in the oral clearance.

In summary, clotrimazole is a potent inhibitor of hepatic cytochrome P450 enzymes in gopher. These characteristics can be exploited in order to design a combination of pesticide with a potent inhibitor to control the gopher population. As such, inhibition of detoxification pathways may provide a logical approach to improving methods of gopher control in the future.

2.2.3. Pilot Study on the Cytotoxicity of Selected Compounds Using Freshly Isolated Gophers Hepatocytes

Background: Liver plays a key role in the metabolism of toxins. In addition to the basic information on detoxification reactions that can be obtained from the isolated cytochrome P450 enzyme system, the use of intact hepatocytes for *in vitro* studies offers an enhanced model which can provide information as to whether the lower metabolic activity translates into a higher or lower potential for hepatotoxicity. A number of different *in vitro* liver models can be used in hepatic toxicity studies.

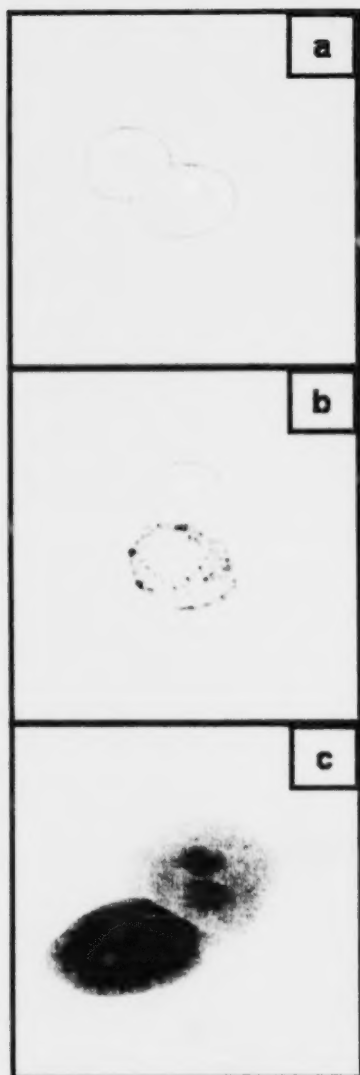
Freshly isolated hepatocytes represent a unique experimental approach to screening various compounds with regard to their cytotoxicity, and it is the most frequently used *in vitro* model for the xenobiotic toxicity study. Accordingly, an exploratory study to evaluate the toxic effects of the selected xenobiotics in the whole cell environment of gopher hepatocytes was conducted.

Materials and Methods (for details see appendix A)

Results

The cytotoxic effect of selected substrates in freshly isolated hepatocytes from gophers was evidenced through morphological examination and from the results of the dye exclusion test. Examples of intact hepatocytes and those showing changes associated with toxicity are presented in Figure 5.

Figure 5. Examples of isolated hepatocytes from gophers.



a) Viable hepatocytes freshly isolated from gopher's liver. The viable cells excluded the trypan blue, which is evident from the characteristic features such intact cellular membranes with shiny silvery color. Original magnification 400x.

b) Hepatocyte showing morphological changes. Cells with multiple cellular membrane protrusions (plasma membrane blebbing) were considered as injured in association with cytotoxicity of administered compounds. Original magnification 400x

c) An example of not viable hepatocytes. Noteworthy is clear staining of nucleus indicative that the cell was unable to exclude trypan blue dye. The figure demonstrates different stages of gopher hepatocytes death. The cell on the right shows mildly blue stained nuclei, which indicates that this cell was in the process of dying, whereas the features seen in the cell on the left with strongly stained nucleus and cytoplasm, indicate that the cell was dead for some time. Original magnification 400x.

Morphological changes such as cell blebbing (Fig 5b) and cell death (Fig. 5c) were observed in the present toxicological studies. At high concentration, all these substrates exhibited a significant cytotoxic effect on the hepatocytes from all three gophers. However, two different types of cellular responses tended to be dominant depending on the substrate: 1) chronic stage with obvious morphological changes (cell blebbing) caused by coumarin and 7-methoxycoumarin, and 2) acute cell death caused by the aniline and its N-methyl derivatives.

The cytotoxicity data for the tested substrates are summarized in Table 6.

Table 6. Mortality of hepatocytes incubated with different concentrations of various substrates (Mean $\pm$ SE).

Toxin concentrations (μ M)	Cell death (%)				
	Aniline	NMA	DMA	Coumarin	7-MC
20 $\times$ K _m	56 $\pm$ 0.58 ^a	46 $\pm$ 4.26 ^a	52 $\pm$ 4.48 ^a	40 $\pm$ 4.51 ^a	42 $\pm$ 4.16 ^a
10 $\times$ K _m	44 $\pm$ 1.20 ^b	40 $\pm$ 3.61 ^{ab}	41 $\pm$ 4.26 ^b	37 $\pm$ 1.15 ^{ac}	33 $\pm$ 1.45 ^b
5 $\times$ K _m	36 $\pm$ 0.88 ^c	37 $\pm$ 3.51 ^{ab}	34 $\pm$ 2.31 ^b	28 $\pm$ 0.58 ^b	33 $\pm$ 0.88 ^b
Control	32 $\pm$ 0.58 ^d	32 $\pm$ 0.58 ^b	32 $\pm$ 0.58 ^b	32 $\pm$ 0.58 ^{bc}	32 $\pm$ 0.58 ^b

NMA= N-methylaniline, DMA= N,N-dimethylaniline, 7-MC=7-methoxycoumarin. The values are means of three replications (SE=standard error). Means were compared using Fisher's LSD, α = 0.05. Means with the same letters are not significantly different.

The present findings clearly showed that treatment with coumarin, aniline, N-methylaniline, N,N-dimethylaniline and 7-methoxycoumarin caused a significant reduction in hepatocyte viability. Interestingly, the cytotoxicity of each compound was further increased by the addition of the inhibitor clotrimazole to the culture medium (Table 7).

Table 7. Comparison of the hepatocyte mortality of different substrates with and without inhibitor and the effect of the inhibitor itself (n=1)

Substrate	Cell death (%)				
	Aniline	NMA	DMA	Coumarin	7-MC
0.625 $\times$ K _m	40	40	39	37	40
0.625 $\times$ K _m + CTZ	47	57	49	58	53
CTZ only	39	39	39	39	39
Control	32	32	32	32	32

NMA= N-methylaniline, DMA= N,N-dimethylaniline, 7-MC=7-methoxycoumarin.

Comments: Liver is the principal organ involved in the biotransformation of orally administered toxins. More than a thousand xenobiotics are potentially hepatotoxic. Freshly isolated hepatocytes, which retain most liver-specific functions, represent a valid and simplified model for predicting the hepatotoxicity of xenobiotics (for review see Gebhardt et al. 2003). They possess the full spectrum of xenobiotic metabolism enzymes, cofactor and cell membrane receptors.

In the present study, hepatotoxicity was observed when selected substrates were incubated with freshly isolated gopher hepatocytes. The cytotoxicity of all selected toxins was found to be

concentration-dependent. When comparing the cytotoxicity of aniline with N-methylaniline and N,N-dimethylaniline; aniline was found to be more toxic. This is consistent with the fact that the detoxification rate of aniline by cytochrome P450 enzymes is much lower than N-methylaniline and N,N-dimethylaniline (for details see above).

Of particular interest are distinctly different types of hepatotoxic effects observed in the present study. Coumarin and 7-methoxycoumarin mainly induced plasma membrane blebbing, a protrusion of cell, shaped like blisters or balloons attached to the cell by a narrow neck, whereas, aniline and aniline derivatives mainly induced cell death with little cell blebbing. Blebbing occurs very quickly after cell damage, which allows it to be a very sensitive indicator of cellular injury. In our study, cell blebbing occurred after two hours of incubation with various toxins. However, it should be noted that cell membrane blebbing is not necessarily a fatal event for the cell, since this kind of change may be reversible (Schwartz et al., 1984).

In this context, the present study provides very important information with potential practical application for screening better means for gopher control. For instance, the toxicity of coumarin or 7-methoxycoumarin can be reversed by removing the source of toxins, whereas, the toxicity caused by aniline, N-methylaniline and N,N-dimethylaniline will be permanent. Considering the bait shyness phenomenon in rodent control, this finding has practical significance. The reason for this is that toxins such as aniline, N-methylaniline and N,N-dimethylaniline will likely cause substantial damage to the hepatocytes of gophers, even following a single exposure to the toxin. However, poison baits containing coumarin or 7-methoxycoumarin, and likely the entire class of coumarin congeners commonly used in pest control, may not be efficient since the toxicity may be reversed once the animal (e.g. gopher) can associate their illness with the bait that will lead to bait shyness.

In conclusion, the results from the isolated hepatocytes indicate that some compounds can cause acute, and likely the terminal effect of cell death when applied in sufficient quantities, whereas other compounds may result in chronic, and possible reversible effects. Overall, a short term *in vitro* cytotoxicity test could be used to help to screen for a wide range of toxins, and to select the *in vivo* starting dose for an acute lethality assay. The use of long-term hepatocyte cultures might be the next procedure since it permits chronic effects to be monitored. Based on observations from our studies, further investigations using the methods employed here are warranted.

General Discussion

The problems caused by gophers have been evident since people began settling on the prairies. Over the years an extensive efforts and large amount of resources have been committed to control the gopher population. Physical methods such as trapping and shooting are time consuming and laborious. Chemical methods, which include various poison-containing baits, are considered to be more effective methods of controlling gopher populations.

However, less than 31% of farmers are satisfied with their gopher control efforts according to a recent report commissioned recently by the government of Saskatchewan (2001 Saskatchewan "Gopher" Survey).

During our own investigations, we counted gophers on a field where no toxins were used (site A) and on a field where toxicological controls were in place (site B). Interestingly, both sites were severely infested with gophers, and we observed no apparent difference in gopher density between the two fields. Both sites contained approximately 75 to 100 gophers in approximately 1 hectare. Our

observations are consistent with the 2001 Survey that suggests that the current chemical methods of gopher control may not be effective. A reason for this is, it could be possible that gophers previously exposed to toxic baits in many areas of the province may have developed resistance to the toxins used, which has caused a significant impediment in the attempts to control gopher populations. Consequently, more effective gopher control methods require development, and chemical methods seem to be the most convenient for the producer.

Currently no scientific data is available to explain this apparent lack of efficiency of the poison control methods in gophers. However, acquired resistance to toxins is a strong possibility, since studies in alternative species indicate resistance in pests is generally an inadvertent outcome of pesticide application, and has caused the loss of many formerly efficacious pesticides (Brown, 1991). Strychnine, which in the past had been an effective compound for gopher control, has been losing its efficiency, likely due to acquired resistance (Matschke et al., 1983; Saskatchewan gopher survey: final report, 2001).

Animals may develop resistance to toxins by three basic mechanisms: 1) behavioral and physiological mechanisms (Devine and Cook, 1998), 2) enzymes involved in resistance (Agosin, 1982), and 3) genetic mechanisms conferring resistance (Feyereisen et al., 1989). Despite the lack of available scientific data regarding behavioral avoidance of the pesticide in gophers, during trapping we observed that gophers approached the peanut butter bait provided in the trap with great caution. The observed behavior (i.e. gophers approached the trap very carefully, looked around, tapped the trap door, went away and came back after sometime) was very consistent among different individuals. Such behavior indicates a highly developed self-preservation instinct.

Regarding the second mechanism, our studies indicated that the content and activity of individual components of the cytochrome P450 system including cytochrome P450, cytochrome b5 and NADPH-cytochrome P450 reductase in liver microsomal preparations were higher in toxin exposed gophers and varied greatly between gophers of different gender. Consequently, gophers exposed to toxins used to control their population had a generally higher detoxification capacity than did naïve gophers. This suggests that gophers previously exposed to toxins may readily develop resistance to chemicals by enhancing the biotransformation potential.

Finally, the resistance to toxins can be transferred genetically, where innately tolerant individuals pass on to their offspring the genes responsible for resistance (Brown, 1991). These changed genes conferring enhanced resistance to a pesticide (e.g. cytochrome P450s) become common genes in the population, since they confer a survival advantage. With continued exposure, a persistent genetic change may occur at the population level (Brown, 1991). It should be noted that in the present study, the sampling process was in the middle of July when most adults went into hibernation (Michener, 1995). Therefore it is reasonable to assume that gophers used in the present study were juveniles. Thus, the enhanced biotransformation potential could have been inherited from the parents previously exposed to the poison.

As mentioned previously, strychnine has been used to control the gopher population in the field where we trapped toxin exposed gophers. Strychnine is metabolized primarily through hydroxylation and N-oxidation by the hepatic microsomal enzyme system (Fujisaki et al., 1994; Mishima et al., 1998). As such, content of cytochrome P450 system in gophers will determine the oxidation extent of strychnine and, consequently, the toxicity of the compound to the animal. Worth mentioning is the fact that strychnine has been identified to be a potent inducer of hepatic P450 metabolizing enzymes in rats (Fujisaki et al., 1994). Similarly, it is possible that the increased content of cytochrome P450

systems found in exposed gophers may have been associated with strychnine induction, which might finally result in extensive metabolism and a corresponding reduction in strychnine toxicity. If this hypothesis is correct, this would be an adaptive response that protects gophers from strychnine toxicity.

From an evolutionary standpoint, xenobiotic dependent induction of cytochrome P450 enzymes in gophers is very important. Cytochrome P450 induction usually enhances detoxification. Hence, under most conditions, induction is a protective mechanism (Klassen, 2001). Furthermore, induction is likely to be advantageous in the survival of a species as it allows enhanced detoxification following exposure to xenobiotics by accelerating the inactivation and clearance of potentially lethal xenobiotic compounds. Other than the toxic compounds used to control the population of gophers, dietary components and environmental pollutants may also cause induction of cytochrome P450 enzymes. In our study, the gopher diets were not extremely variable among individuals, and the compositions of diets was similar for exposed and naïve gophers. Consequently, exposure to chemical control agents likely explains the observed enhancement in cytochrome P450 enzyme system.

In conclusion, a very plausible explanation why the current methods of gopher control are ineffective is the increased capacity of biotransformation acquired upon exposure to toxin. This can be supported by our study. Gophers increased their detoxification capacity by the enhancement of components of the main detoxification system, despite the activity of the enzyme decreasing when gophers were exposed to the toxin and behavioral avoidance to pesticide.

Proposed Strategies to Improve Gopher Control in Saskatchewan

There are three responses to pesticide resistance: 1) increasing the amount of pesticide, 2) increasing the frequency of application, or 3) rotation of pesticides used (NRC, 1986). The first two solutions are self-defeating since they tend to exacerbate the development of resistance. The third solution may ultimately cause its own resistance problems, if there is no basic difference in the mode of action and treatment strategy (Roush, 1981). Therefore, a better alternative for toxicological control of gophers avoiding resistance is needed. Based upon the findings from the present studies, several possible strategies for gopher control should be further researched with emphasis on the following points:

Gender Dependent Differences: The present studies showed that male gophers have higher aromatic hydroxylation, O-demethylation or N-demethylation activity in comparison to female gophers. We can exploit these gender-associated differences for practical gopher control. Since in most cases cytochrome P450 enzyme mediated reactions lead to detoxification of xenobiotics, a compound with relatively high inherent toxicity that undergoes aromatic hydroxylation or demethylation as the principle detoxification pathways may result in enhanced toxicity in female gophers. This may offer an attractive approach to gopher control, since females represent the majority of the adult gopher population, with male to female ratio 1:3 (Michener, 1979; 1995).

Selective elimination of females may effectively decrease gopher population size since the average female gives birth to 6 to 9 juvenile gophers per year (Michener, 1995).

However, it is also well known that some cytochrome P450 enzymes can convert low toxic parent compounds into more toxic metabolites. In this situation, a compound with relatively low inherent toxicity can be metabolized to metabolites that are more toxic than the parent compound. Given the males enhanced xenobiotic metabolism capacity, such compounds will lead to greater toxicity in male gophers. Considering the fact that natural mortality among the male Richardson's ground

squirrel is quite high and the adult sex ratio is about three or four females to one male regardless of the geographic location, year, or population density (Michener, 1979; 1995), further reductions in male populations will reduce the reproductive capacity of the population as a whole.

Cytochrome P450 enzymes may also catalyze activation and detoxification reactions for a given chemical at the same time. In this case, the balance between metabolic activation and deactivation will determine the toxic effect of a xenobiotic chemical, and the results of such a balance will be very complex.

Individual differences in toxin metabolism: The present study identified a considerable degree of individual variability in cytochrome P450 enzyme activity in the gopher population. Variability of biotransformation reactions is very common, the rule rather than the exception. In the present study we identified a sub-group of gophers, which showed lower detoxification capacity than the rest of the population. The sub-population of animals showing poor detoxification activities may represent as much as 25-42% of the population. For this subgroup, if a toxin is used that has inherent toxicity and is detoxified through the same metabolic pathway as aniline, coumarin or 7-methoxycoumarin, then 25%-42% of the gopher population would be more susceptible because of lower inherent detoxification potential. If a toxin that is activated through the same metabolic pathways as aniline, coumarin or 7-methoxycoumarin can be developed, some 58%-75% of the population could be targeted. These apparent "metabolic characteristics" of some individuals could be exploited as a potential **biochemical weakness** for the control of the gopher population.

Kinetic Characteristics of Different Biotransformation Pathways: The present study investigated several different biotransformation pathways including N-demethylation, O-demethylation and aromatic hydroxylation. The results indicated that the K_m values for N-demethylation were higher than aromatic hydroxylation for both naïve and exposed gophers; while, K_m values for O-demethylation were the lowest. On the other hand, the V_{max} value based on cytochrome P450 content for demethylation reactions were higher than aromatic hydroxylation reactions. This knowledge of the kinetic features can also be exploited to provide a better method to control gophers.

For example, N-methylaniline has the highest K_m value, which indicates it can accumulate in the body at high concentration prior to reaching the toxicologically meaningful metabolic biotransformation. A compound with high inherent toxicity and similar kinetics to N-methylaniline would be a desirable rodenticide for gopher control. Conversely, O-demethylation enzymes have the lowest K_m values, which indicate that the toxin will be metabolized at very low concentration. If a compound goes through a similar biotransformation pathway as 7-methoxycoumarin, has a low K_m value and high V_{max} , but is biotransformed to toxic metabolites quickly, the accumulation of such toxic metabolites will be desirable in controlling the gopher population.

The Concept of Toxic Cocktail Rather than Single Pesticides: The present findings indicate that there are significant differences with respect to the kinetics of detoxification of different compounds, as well as large individual variability in metabolic detoxification potential of various toxins. Also, the present study demonstrated that inclusion of inhibitors of detoxification enzymes may be a novel approach to increase the efficacy of toxins used for gopher control.

Taken together, the findings from present project indicate that gopher control based on a single pesticide may not be enough to deal with the wide metabolic polymorphism within the population of gophers. Therefore, a combination of the various toxic agents with a potent inhibitor might be a valuable rationale for the development of a novel means of gopher control in the future. In this

context, the concept of a "pesticide cocktail" comprised of several pesticides, and including some specific inhibitors of biotransformation, is worthy of thorough examination.

Physiological Features of Gophers: A method of control using the concept of "latent or delayed action rodenticide" can be developed taking advantage of the fact that gophers spend the majority of their life underground in hibernation (Michener 1998; Michener, 2002). In this scenario, a highly lipophilic compound with high inherent toxicity can be used. This compound would be sequestered and accumulated in fat tissue during summer, and during hibernation, when fat is mobilized, the compound will be released into the system. Under this circumstance, the accumulation may inevitably progress to a point of high toxicity that would cause gophers to die during hibernation. This concept is very attractive from a wildlife management stand point, because it would decrease the risk of secondary toxicity to the non-target animals.

General Conclusions

The present project provided novel knowledge on major detoxification mechanisms in gophers. From practical stand point, the present study demonstrated that there are several natural features of gophers that can be potentially exploited for management of this pest. Further research and development is warranted. The findings from the present project provided solid base for the second phase of research with emphasis on the following key practical aspects of gopher control method development: 1) work on a generic structure of putative toxins and detoxification inhibitors, 2) preparation of the prototype, and 3) basic toxicological study using cultured hepatocytes followed by field application testing.

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APPENDIX A

Materials and Methods

In Vitro Metabolism of Selected Substrates by the Hepatic Microsomal Cytochrome P450 System in Gophers

General reaction and incubation conditions: Microsomal samples were prepared as described previously (for details see Interim Report #2). For the assays, the preserved stock preparations of microsomes were reconstituted in 0.1M Phosphate buffer (pH 7.4) at the optimal concentration. The optimal concentrations of microsomal proteins for the assays were determined from preliminary experiments (for details see later). Determination of metabolite formation rates provided estimates for the Michaelis-Menten parameters, V_{max} and K_m , for each of the evaluated substrates. Reactions were carried out in 1.5 mL Eppendorf vials. The amount of organic solvents in the final reaction mixtures did not exceed 5% (v/v).

The optimal concentrations of microsomal protein, substrates, and the length of the incubation period to give linear metabolite formation conditions were established for all substrates during method validation. The validation experiments ensured that the rates of product formation were linear with respect to time and protein concentration for all enzymatic activities under study. The reaction mixture contained was: 95 μ l of microsomal suspensions, 5 μ l substrate dissolved in methanol. Following pre-incubation @ 37°C, the reaction was initiated by addition 5 μ l NADPH (10mM) dissolved in phosphate buffer (final concentrations: 0.5 μ M for assays) or 5 μ l of buffer (for blank). The reaction vials were then incubated in a shaking water bath at 37°C open to the ambient air. Eight concentration levels were used for each substrate selected, with the highest concentration being added to the blank (no NADPH). Reactions were terminated by adding 100 μ l of acetonitrile. This termination method effectively stopped the reaction. No changes in concentration of metabolites were observed in the vials even after 24 hours at room temperature. Enzymatic reactions were quantified using authentic metabolite standards. The standards used in our study were prepared using the same conditions as the reaction mixture without substrates as described above

HPLC analysis: After the reaction was terminated, reaction mixtures were centrifuged for 30 min at 14,000 rpm (Microfuge 18, centrifuge, Beckman, Coulter, Germany). The supernatants were then filtered through a 0.45 μ m syringe filter and 10 μ l was injected onto the HPLC. Selected substrates and their metabolites were separated using a reversed-phase column (silica-based C₁₈ (2), LUNA, 150 x 4.60mm, 3 μ , Phenomenex, Torrance, CA), an HPLC system with autoinjector (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) and fluorescence detector (RF-551, Spectrofluorometric detector, SHIMADZU, Columbia, MD, U.S.A.). The methods were validated using standard validation protocol.

Specific experimental parameters for each substrate.

Coumarin aromatic hydroxylation: Coumarin concentrations ranged from 1.56 μ g/ml to 100 μ g/ml. Microsomal protein concentration was approximately 500 μ g/ml, and the reaction mixture was incubated for 5 min. The metabolite standard for coumarin metabolism was 7-OH-coumarin (Fluka, Switzerland). The metabolite and coumarin were separated using 40% methanol as Mobile Phase (MP) at 0.8ml/min flow rate. Detection parameters were: Excitation wavelength (E_x) 325 nm and Emission wavelength (E_m) 445 nm. **Aniline aromatic hydroxylation:** Aniline concentrations ranged from 1.95 μ g/ml to 250 μ g/ml. The microsomal protein concentration was approximately 1mg/ml, and the incubation time of the reaction mixture was 30 min. Metabolite standard for aniline metabolism was 4-aminophenol. The metabolites and aniline were separated by using 50% methanol as MP at 0.8ml/min flow rate. Detection parameters were: E_x 299 nm and E_m 367 nm. **N-methylaniline and N,N-dimethylaniline N-demethylation:** N-methylaniline concentration ranged from 3.91 ng/ml to 500 μ g/ml. The microsomal protein concentration was approximately 0.8 mg/ml. The N,N-dimethylaniline concentration ranged from 0.98 μ g/ml to 125 μ g/ml. Incubation times were 5 min for both substrates. Metabolites standards for N-methylaniline and N,N-dimethylaniline metabolism were aniline and N-methylaniline, respectively. The metabolites and parent compounds were separated by using 70% methanol as MP at 0.8ml/min flow rate. Detection parameters were: E_x 285 nm and E_m 345 nm. **7-Methoxycoumarin O-demethylation:** 7-Methoxycoumarin concentration ranged from 0.20 μ g/ml to 25 μ g/ml. The microsomal protein concentration was approximately 100 μ g/ml, and the incubation time was 5 min. The metabolite standard was 7-OH-coumarin. The metabolite and 7-methoxycoumarin were separated by using 50% methanol acidified with citric acid (pH 4.07) as MP at 0.8ml/min flow rate. Detection parameters were: E_x 299 nm and E_m 367 nm.

Enzyme Kinetics: Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were used to define the enzymatic activity. K_m and V_{max} values were determined by use of nonlinear regression analysis (rate of metabolite formation against substrate concentration). GraphPad Prism 3.03 statistical software for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Both one site binding model and two sites binding model were evaluated. The 95% of confidence interval was evaluated to choose between one-enzyme site and two-enzyme site model. K_m and V_{max} values were determined using one site binding model. For the selected substrates, optimal enzyme reaction conditions and HPLC analysis were determined through method validation procedures.

Study on Inhibition of Selected Detoxification Pathways

Inhibition procedure: The inhibition of cytochrome P450 reactions were investigated using the standard assay condition for each cytochrome P450 substrate as described above. The concentration for the substrate in the final reaction mixture used was 20 $\mu\text{g/ml}$ for dimethylaniline, 50 $\mu\text{g/ml}$ for methylaniline, 50 $\mu\text{g/ml}$ for aniline, and 5 $\mu\text{g/ml}$ for 7-methoxycoumarin. Substrate concentrations for all these compounds were chosen as approaching the K_m value for these substrates determined in the gopher liver microsomes during metabolic studies. Incubations of the assays were performed in the presence of a range of inhibitor (CTZ) concentrations prepared from stock solution of CTZ dissolved in methanol at a concentration of 2mM. The concentration of CTZ was between 0.0001 μM to 100 μM in the final reaction mixture. There was no visible evidence of precipitation of any of these compounds over the concentration range studied. The optimal reaction conditions and HPLC analysis were developed for all substrates as described in metabolism section. The inhibition constant (IC_{50}) were used to define the interactions between the inhibitor and cytochrome P450. IC_{50} values represent the concentration of the inhibitor that was required for 50% inhibition of an enzyme reaction. GraphPad Prism 3.03 statistical software for Windows (GraphPad Software, San Diego, CA) was used for data analysis. Both one site and two sites competition models were tested. Finally, it was decided that IC_{50} values should be determined by using the one site competition model.

Statistical Analyses: All data is presented as means $\pm$ standard error (SE). Statistical analyses were carried out using ANOVA from the microcomputer package Number Cruncher Statistical System (HINTZE, 1995). The means were compared using Fisher's LSD test. Statistical significance was assumed to exist when the probability of making a type I error was less than 0.05.

Pilot Study on the Cytotoxicity of Selected Compounds Using Freshly Isolated Gophers Hepatocytes

Hepatic cell culture preparation: Gophers were trapped from various locations on University of Saskatchewan campus. Liver tissues were harvested immediately after the animals were euthanized according to the protocol described in detail in Chapter 3, and processed at 4°C. The livers were removed and rinsed thoroughly, and then immediately perfused with ice cold 0.1M Phosphate buffer (pH 7.4). A piece of a lobe was sectioned, and further used for hepatocytes isolation. Iscove's Modified Dulbecco's medium (IMD) containing collagenase (0.1 g/L) was used for tissue digestion. The entire procedure was carried out under sterile conditions. The medium was delivered to the tissues via peristaltic pump. After the peristaltic pump (TrisTM, ISCO, Inc., Nebraska, US) was started, a sterilized cannula was introduced into the main vein of the separated lobe. The liver tissue was then perfused for 30 min with collagenase IMD media at a flow rate of 60 ml/min at 37°C. After the digestion, the liver tissue was removed from the apparatus and placed in ice cold 50 ml IMD media. The liver was then minced in the beaker with a pair of sterile scissors to release the hepatocytes into the media. Next, the cell suspension was filtered (cell strainer, sterile, 70 μm Mesh, Falcon, BD#352350, U.S.A.) to separate the hepatocytes. The media was then centrifuged in sterile 15ml centrifuge tubes (500 rpm for 3 minutes at 4°C). The supernatant was discarded and the pellet of hepatocytes was then rewashed with IMD media 3 times followed by re-suspension in IMD media.

Cell viability assay: 4% (w/v) Trypan Blue exclusion test was used to determine the viability of isolated hepatocytes. 5 μl of the 4% Trypan Blue was mixed with 100 μl of the cell suspension, and the mixture then incubated at the room temperature for at least 5 minutes. Cells were then loaded onto a hemacytometer (a counting chamber covered with a cover slide) and counted under a microscope. Cells that did not absorb stain and had membranes intact with shine silver color were regarded as viable cells. Cells with multiple surface protrusions were considered as damaged cells or blebbing cells. Cells with a clearly stained nucleus were counted as dead cells.

Incubation: The cells were diluted to the same cell concentration (about 1×10^5 cell/ml) and were cultured on 96 wells micro-plates. 95 μl of the cell culture and 5 μl of the substrates were then added, mixed thoroughly, and the micro-plates

were covered and incubated at 37°C. Controls were prepared with 95µl cell culture and 5µl methanol and treated identically as the samples were. On a separate plate, substrates (around $0.625 \times K_m$ value for each substrate) with or without the inhibitor (1µM) were added to the cell culture. The plate was then covered and incubated under 37°C. Incubations were terminated after 2 hours, and 5µl of 4% Trypan Blue was added to the 100µl reaction mixture. Following this, the cell culture was examined under the microscope. The cells were counted on randomly selected microscopy fields. For this studies purpose, the numbers of cells showing morphological change as well as dead cells were then enumerated.

Method Validation: In this study, 3 gophers were used to isolate hepatocyte for hepatic toxicity evaluation. The preliminary experiments showed that the optimal cell density and culture substratum were found to be 100 µl of 1×10^5 cell/ml per well of a 96-well plate. Viabilities of hepatocytes of three gophers on the plates were as follow: 90.3% for gopher #1; 85.2% for gopher #2; 84.4% for gopher #3. Next, the hepatocytes were incubated with the highest concentration of each substrate, and the incubation was ended after 2 hours and 4 hours separately. The cell mortality for these two time endpoints was similar. Therefore, 2 hours was chosen as the time factor for incubation for all experiments. The time for dye reaction with cell suspension was evaluated and 5 min was found to be the minimum time for the cell to settle down and for hepatocytes staining. Optimization of the dye reaction duration is critical for cell counting since the cells will settle down as one layer and the dead cells will be clearly distinguished from the viable cells. Cell viability was checked through cross-reference evaluation. In some cases, 3 operators counted the cell variability separately with consistence reached through calculating the mean of the operators cell count.

